The Influence of Lysosomes on Glycogen Metabolism

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Lysosome-rich fractions were isolated from rat liver homogenates. In some fractions the lysosomes were separated from the contaminating mitochondria by the use of discontinuous Ficoll/sucrose gradients. Some glycogen was associated with lysosome fractions. This glycogen was of very large molecular size and of a quite different molecular-weight distribution from that isolated from the cytosol. It is suggested that appreciably more than 10% of cellular glycogen is located within the lysosome.

In type II glycogen-storage disease (Pompe's disease), the massive accumulations of glycogen which are found in the cell were shown (Hers, 1963) to be caused by failure to metabolize intralysosomal glycogen because of the absence of amylo-1,4-glucosidase (acid maltase, EC 3.2.1.20). Patients, who usually die before the age of 2 years, can metabolize extralysosomal glycogen (i.e. cytosol glycogen) normally and therefore are never hypoglycaemic. Death results from severe atrophy of the muscles and perhaps from other causes related to the mechanical disruption of the cell by massively swollen lysosomes. Logic leads us to assume that, where the animal has a normal lysosomal content of α -1,4-glucosidase, then some part of normal glycogen metabolism proceeds through the lysosome. The present paper attempts to show this and also attempts to give a quantitative estimate of the extent of the involvement of normal glycogen metabolism within the lysosome.

The work of Stetten *et al.* (1956) on the total cell glycogen content of liver and muscle-cell tissue from the rat and the rabbit showed that the synthesis and degradation of glycogen is subject to metabolic inhomogeneity, i.e. different molecular sizes of glycogen are metabolized at different rates. The above-mentioned authors extracted the glycogen by methods now known to cause significant degradation of the glycogen. However, the presence of this 'metabolic inhomogeneity' has been confirmed by Geddes (1971) by using extraction methods which produced glycogen whose average molecular weight was at least ten times that obtained by Stetten *et al.* (1956). We show here that the 'metabolic inhomogeneity' is a consequence of the location of high-molecular-weight glycogen within an organelle.

Materials and Methods

Animals

Male white Wistar rats were allowed food and water *ad lib*. They were housed at a temperature of 25°C, and the lighting cycle was strictly controlled (14h light, 10h dark).

Isolation of a mixed lysosomal/mitochondrial fraction from rat liver

Animals weighing about 300g were killed by cervical dislocation. The livers were diced and homogenized in 0.25M-sucrose/20mM-Tris/HCl, pH 7.8 (4ml/g), at 4°C with a Teflon/glass homogenizer. The homogenizer tube had an internal diameter of 19.05mm and the pestle clearance was 0.2mm. To the tube was added approx. 1.5g of liver and 5ml of medium. Eight up-and-down passes were made at a constant pestle speed of 1000 rev./min. According to Ignarro (1971), this buffer helps to maintain the integrity of lysosomes during homogenization.

The homogenate was centrifuged at 760g for 10 min at 4°C (fixed angle rotor, r_{av} 10.8 cm), the pellet was discarded and the cloudy liquid was recentrifuged under the same conditions. The supernatant fluid (fraction S1) was centrifuged a minimum of four times at 16000g for 5 min and 4°C in the same rotor. After each centrifugation the supernatant fluid was decanted (fractions S2–S5) and the mitochondrial/lysosomal pellet (pellet B) suspended in 5ml of homogenizing medium.

Pellet B was further purified by one of the following methods (as shown in Scheme 1).

Method 1A. Essentially linear sucrose density gradients (45ml) were prepared in 50ml centrifuge tubes by repeated freezing and thawing (Baxter-Gabbard, 1972). This method was in routine use, starting with 30% (w/v) sucrose solutions buffered as

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Scheme 1. Purification of the mitochondrial/lysosomal pellet (pellet B)

above and yielding gradients which were essentially linear from 14 to 45% sucrose. The suspension of pellet B (4 ml) was placed on the gradient and centrifuged at 10000 rev./min for 15 min at 4°C (Beckman SW25.2 swinging-bucket rotor): 20 × 2.5 ml fractions were then collected by a simple device (Tan, 1972) and were assayed as described below.

Method 1B. This was used in circumstances where a steeper gradient than that arising from the freeze-thawing technique was required. Gradients were prepared by underlaying nine successively denser sucrose solutions, each of 5.0 ml volume, and leaving them for 4 days at 4°C to permit the sharp discontinuities between layers to diffuse.

Method 1I. By following the procedure of Dingle & Barrett (1969), a heterogeneous gradient was prepared, composed of 35 ml of 14% (w/w) sucrose in 20 mM-Tris/HCl buffer (pH 7.8) layered over 6 ml of 25% (w/v) Ficoll in 14% sucrose/20 mM-Tris/HCl buffer, in turn layered over 6 ml of 54% buffered sucrose, all in a 50 ml centrifuge tube. Suspension of pellet B (7 ml) was layered on top of this gradient and centrifuged (Beckman SW25.2 rotor) either at 7500 rev./min for 30 min (250000 g-min) at 4°C or at 6000 rev./min for 15 min (90000 g-min) at 4°C. Fractions were collected by the method of Tan (1972). (See Fig. 5 below.)

Enzyme assays

Enzymic activities were expressed either as units/litre (μ mol of product formed/min per litre of enzyme solution, at 37°C) or as specific activities (nmol of product formed/min per mg of protein, at 37°C).

The particular enzymes for which assays were performed were chosen because of their varied distribution patterns in studies of lysosomal heterogeneity (Beaufay, 1969; Davies, 1975). No attempt was made to distinguish between the various possible populations of lysosomes in the liver (Davies, 1975).

Acid phosphatase. Acid phosphatase activity was assayed with *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO, U.S.A.) by the method of O'Brien *et al.* (1968). Tissue preparations were

diluted considerably before assay, so turbidity was not a problem. Dilution also eliminated the inhibitory effect of sucrose (Beaufay, 1972).

Any inherent errors caused by the use of *p*-nitrophenyl phosphate (it may also be hydrolysed by microsomal glucose 6-phosphatase; Beaufay, 1972) were eliminated (i) by making no quantitative use of the apparent activities to gauge lysosomal enhancement and (ii) by running parallel enzyme assays (β -glucuronidase, arylsulphatase or α -glucosidase) to establish the segregation of lysosomal activity. However, it should be noted that none of the enzymic activities mentioned are exclusively lysosomal (e.g. de Duve *et al.*, 1955).

β -Glucuronidase. This was assayed by the method of Fishman *et al.* (1948), with phenolphthalein glucuronide (Sigma) as substrate.

Arylsulphatase. The method of Roy (1960) was used, except that 4-nitrophenyl sulphate (Calbiochem, San Diego, CA, U.S.A.) replaced the original substrate, 4-nitrocatechol sulphate.

α -Glucosidase. The method of Den Tandt (1972) was used.

Monoamine oxidase. Monoamine oxidase activity was used as a mitochondrial marker and was assessed by micro-radioassay (McCaman *et al.*, 1965) with 5-hydroxy[2-¹⁴C]tryptamine (The Radiochemical Centre, Amersham, Bucks., U.K.).

Protein determination

Total protein was assayed by the method of Lowry *et al.* (1951), with Folin-Ciocalteu reagent from BDH Chemicals Ltd. (Poole, Dorset, U.K.) and bovine serum albumin (Sigma) as a standard.

Release of lysosomal enzymes

In an attempt to obtain maximal lysosomal enzyme activity in the enzymic assays, a comparison was made between thermal and the much milder hypo-osmotic disruption of the lysosome. The technique of thermal enzymic release was based on that described by Brown & Schwartz (1969). A portion of the enzyme solution in 0.25 M-sucrose/5 mM-sodium acetate buffer,

Fig. 1. Comparison of lysosomal-enzyme labilization methods

Assays for (a) acid phosphatase, (b) β -glucuronidase and (c) α -glucosidase were performed (see the Materials and Methods section): (i) on the liver homogenate after removal of the nuclei and cellular debris by centrifugation (fraction S1); (ii) on the supernatant (fraction S2) after compaction of the lysosomal/mitochondrial pellet by centrifugation of fractions S1 and S3 after centrifugation of the resuspension of the pellet remaining in fraction S2; (iii) on the final suspension of the lysosomal/mitochondrial pellet (pellet B). The enzymes were released either by exposure to hypo-osmotic conditions (□) or by thermal labilization (▨) (see the text).

pH 6, was incubated at 45°C for 90 min. After centrifuging at 4°C (300 000g-min) a sample of the supernatant was used for enzymic assay. Hypo-osmotic release of enzymes was performed by diluting the enzyme fractions 10- or 20-fold with water, then leaving them at 0°C for approx. 30 min.

A comparison of the results obtained with these two methods is shown in Fig. 1, and indicates first that, regardless of the method used, acid phosphatase has greater specific activity than either β -glucuronidase or α -glucosidase. This is in agreement with the results of Tappel (1969). Secondly, the changes in specific activities relative to the original supernatant activity of all three enzymes parallel one another during the purification after hypo-osmotic release, but there are major anomalies after thermal labilization. Significant enhancement of enzymic activity in pellet B is shown only by β -glucuronidase. Presumably this discrepancy is related to the mode of rupture of the lysosomal membrane at 45°C. A large proportion of the acid phosphatase and α -glucosidase may not have been released from the membrane before centrifugation, whereas the β -glucuronidase is considerably more labile. Therefore for the purpose

of the present paper the method of Brown & Schwartz (1969) was considered unsatisfactory and all subsequent releases of enzymes were performed hypo-osmotically. The presence of Triton X-100 or freezing and thawing (Gianetto & de Duve, 1955; Wattiaux & de Duve, 1956) did not increase enzymic activity.

Glycogen extraction

When total glycogen (i.e. cytosol and organelle glycogen) was to be extracted, the whole liver was placed in liquid N₂ immediately after removal from the animal, to minimize degradation (Geddes & Rapson, 1973) (time from cervical dislocation to immersion of the excised liver in liquid N₂ averaged 6 s). If subfractionation of the tissue had already taken place, the freezing was omitted.

The tissue or tissue subfraction was homogenized in 4 vol. of 45% (w/v, final concn.) phenol for 4 min at 25°C and then stirred gently for 1 h. The upper aqueous layer containing glycogen and RNA was removed (Kirby, 1956; Laskov & Margoliash, 1963). The RNA was removed during overnight dialysis of the aqueous layer by the addition of ribonuclease

(Koch-Light Laboratories, Colnbrook, Bucks., U.K.) to the contents of the dialysis sac (approx. 1 mg/10 ml).

Fractionation of glycogen

Purified and concentrated (approx. 12 mg/ml) glycogen solutions (4 ml) were placed on essentially linear sucrose gradients [approx. 10–50% (w/v) sucrose, 45 ml (Baxter-Gabbard, 1972)] and centrifuged in a Beckman Spinco SW25.2 swinging-bucket rotor at 15000 rev./min for 30 min at 20°C. Fractions (approx. 4 ml) were removed from the bottom of the tubes by using the device of Tan (1972) and were analysed for glycogen by using an iodine-iodide reaction (Krisman, 1962; Drochmans, 1966). This colorimetric assay was unaffected by the concentration of the sucrose. Sucrose concentrations were determined by refractometry.

Results and Discussion

Differential centrifugation, the classic method for isolating lysosomes (de Duve *et al.*, 1955), has the merits of speed and easy reproducibility. We assumed that these advantages might outweigh the disadvantages of the inevitable damage to subcellular

particles caused by the use of high *g* values (Wattiaux, 1974). Thus our first experiments were carried out with lysosomal fractions obtained essentially by the method of de Duve *et al.* (1955). However, it was necessary to wash the mitochondrial/lysosomal pellet (pellet B) at least four times to remove cytosol glycogen (Fig. 2). Cytosol, i.e. extralysosomal, glycogen could be decreased to less than 1% of its value in fraction S1 by this procedure. However, when the pellet was put into a hypo-osmotic medium for 30 min, glycogen was released from the subcellular organelles. Assays of the various fractions demonstrated a concentration of lysosomal enzymes in pellet B, as indicated by their relative specific activities (Fig. 3). The specific activity of the pellet was more than doubled by the two extra washings (cf. Fig. 1, which shows that the specific activity of pellet B is approx. three times that of fraction S1). No explanation for this phenomenon is offered. Recovery of glycogen at the end of the subcellular fractionation (Table 1) averaged about 80% of that found in the original homogenate. This loss is easily understood because of the lability of glycogen in the presence of lysosomal α -glucosidase, the additional presence during the preparation of cytoplasmic phosphorylase and serum amylase, and the time taken to isolate pellet B, normally about 50 min. As there has been shown (Geddes & Rapson, 1973), glycogen, in the intact post-mortem animal, when the cellular glycogen is presumably not

Fig. 2. Glycogen contents during preparation of the lysosomal/mitochondrial pellet

Glycogen contents were measured relative to that of the cell-free homogenate (fraction S1) during the preparation of the lysosomal/mitochondrial fraction (see the text). Values are means for seven separate preparations and the error bars indicate the maximum spread of values.

Fig. 3. Localization of lysosomal enzymes
The specific activities of acid phosphatase (□) and β -glucuronidase (■) were measured during the preparation of the lysosome/mitochondria fraction (see the text) and expressed relative to those of the cell-free homogenate (fraction S1).

exposed to the serum amylase, is significantly degraded over even much shorter periods than this.

Glycogen isolated from the whole homogenate, from the supernatant of a pellet which had been washed four times (fraction S5), and from the disrupted B fractions were fractionated on linear sucrose gradients; from these data, calculations of the type described by Martin & Ames (1961) were performed for each fraction to yield sedimentation

coefficients, which were combined with the diffusion coefficients of the same fractions obtained by laser-dynamic-light scattering (intensity-fluctuation spectroscopy) (Chu, 1974) in the Svedberg equation. The resultant molecular-weight distributions are shown in Fig. 4 [details of this method are given in the following paper (Geddes *et al.*, 1977)]. The obvious similarities between the distributions for the homogenate glycogen and the cytosol glycogen are as expected. The glycogen isolated from the pellet, by contrast, exhibits a very unusual distribution, with a predominance of high-molecular-weight material; in fact 70% of the glycogen has a mol.wt. of over 500×10^6 . For glycogen obtained from the whole cell, this value is 18% (average of 30 samples, see Fig. 6).

Further, it has been shown (Geddes & Rapson, 1973) that degradation of glycogen by cellular enzymes proceeds rapidly and causes the high-molecular-weight material to be depleted first. Thus it may be safely concluded that the estimate of 70% for the percentage content of high-molecular-weight glycogen from that isolated from the lysosomal pellet is conservative and may approach 100% *in vivo*.

The variation of percentage glycogen content in the organelle due to normal biological variation

Table 1. *Recovery of glycogen during preparation of the mixed lysosomal/mitochondrial fraction*

Cytoplasmic glycogen is the total glycogen recovered in fractions S2–S5 inclusive. Organelle glycogen is glycogen released from pellet B after hypo-osmotic shock. Total glycogen is the glycogen measured in fraction S1. Results are means $\pm$ s.d. for six separate preparations.

Glycogen ratio	Ratio expressed as a percentage
Cytoplasmic+organelle	78.0 $\pm$ 17.0
Total	
Organelle	9.5 $\pm$ 2.2
Cytoplasmic	
Organelle	6.6 $\pm$ 1.8
Total

Fig. 4. *Molecular-weight distributions of cellular glycogens*

Glycogen isolated from the intact cell (○) or the soluble cytoplasm (fractions S1–S5) (●) or the lysosomal/mitochondrial pellet (pellet B) after exposure to hypo-osmotic solution (■) was fractionated on a sucrose density gradient, and the results were expressed as a molecular-weight distribution [see the following paper (Geddes *et al.*, 1977)]. All concentrations were normalized in average weight units with respect to fraction number (not molecular weight).

between animals will depend on, in addition to this simple enzymic degradation, the individual degree of hormonal excitement of the animal at the time of death and unavoidable variations in the efficiency of homogenization. However, it is clear that there is a significant association of glycogen with pellet B, which is enriched with lysosomes and mitochondria.

Three conclusions may be drawn from this experiment, namely: (i) that some glycogen is associated with a subcellular organelle; (ii) that this glycogen is of predominantly, perhaps exclusively, high molecular weight; (iii) that the subcellular organelle is possibly the lysosome.

Further, it is clear that the multiple resuspensions of the lysosomal pellet during the preparation eliminates the possibility that the glycogen is occluded in the preparation and makes surface adsorption unlikely.

However, there is only a small relative increase in the specific activity of the lysosomal enzymes. As shown in Fig. 3, the increase is approx. ninefold, and the predominant organelles in the preparation will still be mitochondria. It was therefore decided to pursue other methods for separation of lysosomes from mitochondria, even though the longer preparation times might well permit more extensive degradation of glycogen. Isopycnic banding proved unsatisfactory, but our modification of the procedure reported by Dingle & Barrett (1969) gave good separation.

Centrifuging at 7500 rev./min for 30 min (250 000 g-min) has the advantage of very good separation of lysosomes and mitochondria but the disadvantage of significant contamination of the Ficoll layer with extra-organelle glycogen. Centrifugation at 6000 rev./min for 15 min (90 000 g-min) eliminated significant contamination of the Ficoll layer with extra-organelle glycogen, but only about half the mitochondria descended into the 54% sucrose layer. Results are summarized in Table 2 and Fig. 5. Table 2 indicates that nearly 10% of the total glycogen is associated with the Ficoll layer and hence with the lysosomes, in confirmation of the above experiments. Also, only

Fig. 5. Distribution of marker enzymes in a discontinuous sucrose/Ficoll gradient

The distributions of the specific activities of lysosomal and mitochondrial marker enzymes were measured after centrifugation of cell-free homogenate on a discontinuous sucrose/Ficoll gradient (see the text) at (a) 90 000 g-min or (b) 250 000 g-min. ○, Acid phosphatase; ●, β -glucuronidase (5 $\times$ activity); ■, monoamine oxidase. Fractions (first 15 $\times$ 2 ml, then 6 $\times$ 4 ml) were collected. Fraction numbers are plotted from right to left, since the fractions were collected from the bottom of the centrifuge tube.

very small amounts of cytosol glycogen descend into the Ficoll layer during the centrifugation, and this amount does not depend on the presence of RNA.

Table 2. Glycogen distribution in the discontinuous Ficoll gradients used for lysosome purification (Method II) under the standard conditions used

Samples placed on the gradient were either cell-free homogenate (fraction S1) or a solution of isolated glycogen before ('RNA-glycogen') or after treatment with ribonuclease.

Sample	$10^{-4} \times g$ -min	Position in gradient ...	Glycogen (% of total)		
			Sucrose (14%)	Ficoll	Sucrose (54%)
Homogenate	9		90.5	9.4	0.2
RNA glycogen	9		99.5	0.5	0.0
Glycogen	9		99.7	0.3	0.0
Homogenate	25		84.6	11.0	4.5
Glycogen	25		97.3	2.2	0.5

Further, as shown in Table 3, when this glycogen is removed from the Ficoll layer by the phenol/water extraction method and compared with that extracted from the 14% sucrose layer, again the organelle-associated material contains significantly more high-molecular-weight material than is normal (Fig. 6); 30% of the glycogen from the Ficoll layer and 13% of the glycogen from the 14% sucrose layer is of mol.wt. greater than 500×10^6 . The decreased amount of high-molecular-weight material found in this experiment is clearly related to the longer extraction process before glycogen is removed from contact with its enzymic environment. Further, simple calculations based on an average sedimentation coefficient for the high-molecular-weight glycogen of 5000S (approx. 1×10^9 daltons) and taking account of

the viscosity of the 14% sucrose layer show clearly that material even of this size could not have progressed down the gradient in the time of the centrifugation by itself. To sediment the distance down the centrifuge tube into the Ficoll layer, it must have been associated intimately with a particle.

Conclusion

The experiments described above show that glycogen is associated with lysosomes (or lysosome-like organelles). It seems likely that some glycogen is segregated into an autophagic vacuole which then fuses with a primary lysosome to form a secondary lysosome. The amount of cellular glycogen so associated is at least 10%, but is probably significantly greater when the rapid post-mortem degradation of glycogen (Geddes & Rapson, 1973) is considered. Further, this post-mortem degradation attacks preferentially the high-molecular-weight material and will cause the percentage of high-molecular-weight glycogen to decrease markedly over the time period normally involved in performing experiments of the type described above. This intralysosomal localization of glycogen is clearly of more significance in normal carbohydrate metabolism than has previously been suspected. Little account, if any, is taken of the lysosome in discussion of carbohydrate metabolism in most textbooks (e.g. Lehninger, 1975; Mahler & Cordes, 1972). Further, from experiments with radioactive-tracer incorporation into glycogen (R. Geddes & G. C. Stratton, unpublished work), the turnover of high-molecular-weight glycogen does not

Table 3. *Percentage of small and large glycogen recovered from the three different portions of the discontinuous Ficoll gradient (Method II)*

Large glycogen is arbitrarily classified as that of mol.wt. greater than 500×10^6 . Calculations are based on the data from which Fig. 6 was constructed. The total glycogen refers to the mean for 30 different preparations.

Origin of sample	Glycogen (% of total)	
	Small	Large
Total	82	18
Sucrose (14%)	87	13
Ficoll	70	30

Fig. 6. *Molecular-weight distributions of cellular glycogens*

The conditions used to obtain the organelle and cytosol glycogens were the same as those for Fig. 5(b) (Method II; see the Materials and Methods section). Molecular-weight distributions were measured as in Fig. 4. Cytosol glycogen (■) was isolated from the 14% sucrose layer, organelle glycogen (●) from the Ficoll layer. The distribution for glycogen isolated from the intact cell (mean for 30 separate preparations) is shown for comparison (□, Geddes *et al.*, 1977).

normally exceed the turnover of low-molecular-weight glycogen, and in fact appears to be far slower. It therefore seems that the lysosome acts as a storage unit for high-molecular-weight glycogen. The physiological benefit of this would be that, in stress situations, such as starvation, when lysosomes normally rupture, this causes a final energy store to be made available to the whole animal.

At the polysaccharide level these results clearly provide an explanation for the metabolic inhomogeneity pointed out by Stetten *et al.* (1956) and demand further investigations of the possible involvement of lysosomes with carbohydrate metabolism in other tissues, e.g. the chondrocytes of epiphyseal tissue have been reported to be full of glycogen.

With respect to Pompe's disease, these results merely confirm the seriousness of the lack of lysosomal α -1,4-glucosidase in the animal. Since this enzyme is the 'release mechanism' within the lysosome that allows turnover of the stored high-molecular-weight glycogen, absence of the enzyme will cause the stores of glycogen to increase, as has been observed (Hers, 1963).

The question which remains unanswered is: what is the nature of the stimulus that causes the lysosomes to recognize and engulf large glycogen particles while leaving the smaller particles in the cytosol? The stimulus must be physical rather than chemical, since small glycogen is chemically indistinguishable from its large counterpart.

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